

Vitellogenin Induction by Xenobiotic Estrogens in the Red-eared Turtle and African Clawed Frog

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Many environmental pollutants have estrogenic activity in animals. Xenobiotic estrogens include many pesticides and industrial chemicals that bioaccumulate. The impact of these common pollutants on the reproductive success of wildlife may be considerable, particularly in threatened or endangered species. This research examined the use of plasma vitellogenin in males as a biomarker for estrogenic xenobiotics in reptiles and amphibians. Adult male turtles (*Trachemys scripta*) and frogs (*Xenopus laevis*) were given ip injections of estradiol-17 β (E_2), diethylstilbestrol (DES), or *o,p'*-DDT (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene) daily for 7 days, and plasma was collected on day 14. The estrogenic activity of each compound was determined by measuring the induction of plasma vitellogenin. Vitellogenin was identified by precipitation, electrophoresis, Western blot, and enzyme-linked immunosorbent assay (ELISA). In both species, estradiol and DES treatments induced the most vitellogenin, whereas DDT treatments induced smaller amounts of vitellogenin in a dose-dependent fashion. These data indicate that induction of plasma vitellogenin in males may be a useful biomarker of xenobiotic estrogen activity in wild populations of reptiles and amphibians. — Environ Health Perspect 103 (Suppl 4):19–25 (1995)

Key words: estrogen, xenobiotic, diethylstilbestrol, DDT, turtle, frog, vitellogenin

Introduction

Xenobiotic estrogens in the environment pose an insidious risk to both wildlife and humans. By disrupting reproduction and developmental processes, they may impair not only the individuals exposed, but have lasting influence on their offspring as well. Xenobiotic estrogens include many herbicides, fungicides, insecticides, and industrial chemicals. At least 45 chemicals or their metabolites have been identified as endocrine disrupters (1). The impact of these common pollutants on the reproductive success of wildlife may be considerable. Due to their lipophilic nature and persistence in the environment, many of these compounds bioaccumulate (2). Therefore,

those most affected will be those in the upper food chain or aquatic species that are exposed to high doses due to toxic accumulation in aquatic ecosystems.

A rapid, sensitive, and inexpensive test to screen wildlife for the effects of estrogenic chemicals is greatly needed. This test should be one that demonstrates not merely the presence of a compound in the tissues or body fluids, but measures a biological effect. An ideal test should meet several criteria, specifically *a*) have widespread applicability across many animal groups; *b*) require a small and easily obtainable sample without causing undue harm or distress to the animals; *c*) measure a physiological response to xenobiotic compounds; *d*) measure a response through known biochemical pathways; *e*) be responsive to a large class of estrogenic chemicals; and *f*) be quantifiable.

One of the most important and sensitive responses to estrogen is the induction of protein transcription and translation (3,4). Particularly well known among these responses is estrogenic induction of the lipoprotein vitellogenin, expressed in all oviparous and ovoviviparous vertebrates (5,6). This makes testing for vitellogenin useful as an indicator of estrogenic activity over a wide range of vertebrate groups. Further, the mechanism has been studied in detail as a model of estrogen action (4–13). Vitellogenin is produced by cells in the liver in response to estrogen. The response is only elicited if an estrogenic compound binds to and activates estrogen receptors in

hepatocytes. In females, vitellogenin is transported in the circulatory system to the ovaries, where it is incorporated into the developing ovarian follicles as yolk.

Males normally have no detectable production of vitellogenin due to their normally low levels of endogenous estrogens (13–18). However, their liver is capable of synthesizing and secreting vitellogenin into the blood in response to exogenous estrogen stimulation (6,17–25). The response is not as rapid or as strong as in females that are exposed to the same concentrations of estrogen (18). However, since males normally have no vitellogenin, the expression of any vitellogenin serves as an ideal biomarker for xenobiotic estrogenic stimulation. Vitellogenin expression has been used successfully for identification of exposure to environmental estrogens in fish, including wild populations (26,27), and under laboratory (28) and *in vitro* conditions (29,30).

This study was undertaken to determine if plasma vitellogenin is inducible by xenobiotic estrogens in male turtles and frogs, therefore making it a potential biomarker of xenobiotic estrogen exposure in reptiles and amphibians. Turtles and frogs were exposed to *a*) natural estrogen, estradiol-17 β (E_2); *b*) a well-known artificial estrogen, diethylstilbestrol (DES); and *c*) an important environmental estrogen, the pesticide *o,p'*-DDT. Measurable vitellogenin was induced in both turtles and frogs, indicating that vitellogenin may be

This paper was presented at the Conference on Environmentally Induced Alterations in Development: A Focus on Wildlife held 10–12 December 1993 in Racine, Wisconsin.

We acknowledge the efforts of Dr. Malcolm Powell for assistance with polyclonal antibody production; Dr. Kenneth Goodrum for assistance with ELISAs; Dr. Kyle W. Selcer for discussions and comments; Biotechnologies for the Ecological, Evolutionary and Conservation Sciences Program for plasma radioimmunoassays; and Jane Perkins and Sue Simon for assisting with animal care, blood collection, and data analyses. This research was supported in part by Ohio University College of Osteopathic Medicine, the Department of Biological Sciences, the Office of Animal Research, and a grant from Ohio University Research Committee.

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useful as a biomarker for xenobiotic estrogen exposure in amphibians and reptiles.

Materials and Methods

Species

Adult red-eared turtles (*Trachemys scripta*) were purchased from a commercial dealer (Lemberger; Oshkosh, WI). The turtles were housed in large Rubbermaid tubs (1.25 m long × 0.75 m wide × 0.75 m deep) at 21°C. Each tub was supplied with a basking ramp mounted 0.3 m below 150-watt flood lamps. This allowed the animals to thermoregulate their body temperature. Food (Wardley Pond-Ten; Secaucus, NJ) was provided *ad libitum* 3 days per week.

Adult male African clawed frogs (*Xenopus laevis*) were purchased from a commercial dealer (Sullivan; Nashville, TN). The frogs were divided randomly into groups of 5 and housed in 5-gal glass aquaria in 10 cm of water maintained at room temperature (21°C). Food (Wardley Pond-Ten) was provided 3 days per week, and the water was changed the following day.

Chemicals

Estradiol-17 β (E₂; Sigma, St. Louis, MO), diethylstilbestrol (DES; Sigma), and *o,p'*-DDT (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene; AccuStandard, New Haven, CT) were dissolved in corn oil. Placebos consisted of pure corn oil. Volumes of all injections were equilibrated across treatment groups for each species.

Production of Turtle Vitellogenin for Antibody Production

Six adult female turtles were injected daily with 1 μ g/g E₂ for 7 days, and on alternate days until day 21. Blood was collected from the caudal vein of each turtle using a 1-ml syringe and a 26-gauge needle (31). Whole blood was centrifuged in heparinized tubes for 2 min at 14,000g, and the plasma was separated and frozen at -20°C. Vitellogenin was purified by precipitation and checked for purity by electrophoresis.

Purification of Vitellogenin

The protocol for purification of vitellogenin was modified from that described by Wiley (32). Two-hundred fifty microliters of plasma sample were gently mixed with 1 ml of 20 mM Na₂EDTA and 80 μ l of 0.5-M MgCl₂ and centrifuged at 2500g for 15 min. The supernatant was discarded, and the pellet containing vitellogenin was redissolved in 150 μ l of 1 M NaCl, 50 mM Tris-HCl (pH 7.5) and then cen-

trifuged at 2500g for 30 min. The supernatant was removed to another centrifuge tube, and then vitellogenin was precipitated with 1.24 ml distilled H₂O and centrifuged at 2500g for 15 min. The resulting pellet of purified vitellogenin was redissolved in 1 M NaCl, 50 mM Tris-HCl (pH 7.5) and stored at -20°C. Purification of frog vitellogenin was performed using 50 μ l of plasma and proportionately reduced quantities of other reagents. Quantification of vitellogenin was accomplished using Lowry reagent (BioRad, Melville, NY).

Polyclonal Antibody Production and Purification

Turtle (*T. scripta*) vitellogenin was purified as above and used to produce polyclonal antibodies in rabbits. Turtle vitellogenin (0.50 mg in 1 ml of 1 M NaCl, 50 mM Tris-HCl, pH 7.5) was mixed with 1 ml of Freund's complete adjuvant and injected intradermally at four to six sites along the back of rabbits using a 20-gauge 1.5-inch needle. Using the above protocol the rabbits were boosted twice with Freund's incomplete adjuvant at 3-week intervals. The rabbits were bled by cardiac puncture under deep anesthesia. The blood was allowed to clot at 4°C overnight to separate the serum, which was then stored at -80°C.

Antivitellinogen antibodies were purified using a procedure modified from Harlow and Lane (33). A 1-ml column was prepared by swelling 0.3 g Sepharose-4B-CNBr in 1 mM HCl for 15 min, rinsing with 70 ml of 1 mM HCl, and washing 3 times with 1 ml coupling buffer (0.5 M NaPO₄, pH 7.5). Precipitation-purified vitellogenin was dissolved in coupling buffer (20 mg/2 ml), added to the Sepharose column, agitated overnight, and rinsed twice with coupling buffer. The column was washed once with 1 M NaCl, 0.05 M NaPO₄ (pH 7.5) and incubated overnight, with gentle rocking, at room temperature with 10 vol of blocking buffer (100 mM ethanolamine, pH 7.5). The column was washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) and merthiolate added to 0.01% and stored at 4°C until use.

Before use, the column was rinsed with 10 ml of 10 mM Tris buffer (pH 7.5) and flushed with 10 ml of 100 mM glycine buffer, pH 2.5, followed by 10 ml of 10 mM Tris buffer pH 8.8. The column was then washed with 10 ml of 100 mM triethylamine (pH 11.5), followed by 10 ml of 10 mM Tris, pH 7.5.

Following column preparation, polyclonal antiserum specific to vitellogenin was diluted to 10% in 10 mM Tris, pH 7.5 and passed through the column 3 times with a flow rate of 1 ml/min. The column was washed with 20 ml of 10 mM Tris, pH 7.5 and then 20 ml of 500 mM NaCl, 10 mM Tris-buffered saline (pH 7.5). The column was eluted with 20 ml of 100 mM glycine buffer, pH 2.5, and the antibodies collected in a tube containing 1 ml of 1 M Tris, pH 8.8. The column was washed with 10 mM Tris, pH 8.8, the column eluted again with 10 ml of 100 mM triethylamine, pH 11.5. Antibodies were collected in a tube containing 1 ml of 1 M Tris, pH 8.8. The vitellogenin antibody fractions were combined and dialyzed against PBS with 0.02% sodium azide.

Specificity of purified antivitellogenin antibody was tested against whole plasma, precipitation-purified vitellogenin, a protein of 214 kDa (the presumptive vitellogenin identified electrophoretically) electroeluted from 5% PAGE, and bovine serum albumin (BSA). Antivitellinogen antibodies were tested by enzyme-linked immunosorbent assay (ELISA) and Western blot to determine cross-reactivity. Controls included omission of protein antigen, omission of primary antibody, and omission of secondary antibody. Only batches of purified antivitellogenin antibody that exhibited high affinity for vitellogenin and minimal cross-reactivity with other antigens were used in immunodetection.

One-dimensional Polyacrylamide Gel Electrophoresis

Whole plasma or purified vitellogenin samples were solubilized in 10 mM Tris buffer (pH 7.4) containing 2% (w/v) sodium dodecyl sulfate (SDS) and 10% (v/v) 2-mercaptoethanol and separated by molecular weight using one-dimensional SDS polyacrylamide gel electrophoresis (1D-SDS-PAGE). Separation gels were made from 5% T (total acrylamide) to facilitate analyses of high molecular weight proteins (34). Plasma samples or purified vitellogenin were loaded on to a discontinuous PAGE apparatus, then run at 200 V for 35 min or until complete. The gels were fixed and stained with Coomassie blue (34) and silver (35). Molecular weights were determined using BioRad high molecular weight standards and calculation of R_f values (36). Vitellogenin was quantified from 1D-SDS-PAGE of plasma using a BioRad GS-670 imaging densitometer to digitize the images. The concentrations of vitellogenin (band densities)

were calculated by plotting the band absorbance and integrating the area under the curve for vitellogenin (BioRad Molecular Analyst software). Comparisons of treatment groups were performed using Kruskal-Wallis one-way analysis of variance on ranks (ANOVA: $p < 0.05$). Multiple means comparisons were performed using the Student-Newman-Keuls method (37).

Western Blotting

To identify vitellogenin immunologically, whole plasma proteins or precipitation purified vitellogenin were transferred to polyvinylidene difluoride (PVDF) membranes immediately following 1D-SDS-PAGE. Briefly, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) (38) immediately following electrophoresis, and the proteins were transferred to a PVDF membrane under a 15V electrical field for 1 hr. After transfer, the PVDF membrane was equilibrated in Tris-buffered saline (TBS; 25 mM Tris, 0.3M NaCl, pH 7.4) for 15 min, blocked with 5% (w/v) powdered milk in TBS (2 hr), and incubated overnight with primary antibodies (diluted 1:50,000 in 5% (w/v) powdered milk in TBS) specific for vitellogenin. The transfer membrane was washed in TBS (3×5 min) and incubated for 2 hr with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in 5% powdered milk in TBS. The PVDF membrane was washed again in TBS (3×5 min), and incubated at 37°C in 0.1 g/l nitro blue tetrazolium (NBT), 0.05 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 2 mM $MgCl_2$ in 0.1M Tris buffer (pH 8.8 for 15 min) for localization of reactive proteins.

Induction of Vitellogenin in Turtles with E_2 , DES, and o,p' -DDT

Adult male turtles were ranked by mass and divided into experimental and control groups to equalize specimen sizes among treatments. The treatment groups consisted of animals treated with 1 μ g/g E_2 , 1 μ g/g DES, 250 μ g/g o,p' -DDT, 1 μ g/g o,p' -DDT, and oil only as control. Each compound was dissolved in 0.1 ml corn oil and injected ip. The treatments were given daily for 7 days. Plasma was collected on day 14 from the caudal vein of each turtle using a 1-ml syringe and a 26-gauge needle (31). Whole blood was spun in heparinized tubes for 2 min at 14,000g, and plasma was separated and frozen at -20°C. This treatment protocol with

exogenous E_2 has been shown to produce circulating concentrations of serum E_2 in turtles of approximately 333 pg/ml (Selcer and Palmer, unpublished data), which is equivalent to E_2 concentrations in females during natural vitellogenesis.

Induction of Vitellogenin in Frogs with E_2 , DES, and o,p' -DDT

Twenty-five adult male frogs of matched size were randomly assigned into treatment groups of five and housed in separate 5 gal aquaria with 10 cm of water. The treatment groups consisted of animals treated with 1 μ g/g E_2 , 1 μ g/g DES, 250 μ g/g o,p' -DDT, 1 μ g/g o,p' -DDT, and oil only as control. Compounds were dissolved in 40 μ l of corn oil and injected ip. Injections were given daily for 7 days, and frogs were bled by cardiac puncture on day 14. Whole blood was centrifuged in heparinized tubes for 2 min at 14,000g, and plasma separated and frozen at -20°C.

ELISA

ELISA (enzyme-linked immunosorbent assay) was used to determine relative levels of vitellogenin in plasma samples. Ten-microliter plasma samples and 10 μ l excess BSA were each diluted with 40 μ l TBS (25 mM Tris, 0.3M NaCl, pH 7.4). The diluted plasma solutions were added to individual wells of a polystyrene microtiter plate, and the BSA solution was added to a single well as a control; and the plate was incubated overnight at 4°C. Plates were washed three times with TBS-Tween (TBS plus 0.2% Tween 20) and blocked with 150 μ l Blotto-Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in TBS) for 2 hr. The plates were washed three times with TBS-Tween, and 50 μ l antivitellogenin antibodies diluted 1:20,000 in Blotto-Tween were added to each well. The plates were then incubated for 2 hr at room temperature. The plates were thoroughly washed four times with Blotto-Tween and incubated for 2 hr with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in Blotto-Tween. After thoroughly washing each well with TBS-Tween 4 times, the plates were developed in 0.1% (w/v) p -nitrophenyl phosphate (PNPP) in developing buffer (0.1 M NaCl, 0.1 M Tris, 5 mM $MgCl_2$, pH 9.5) and incubated for 15 min at room temp. The reaction was stopped with 50 μ l of stop buffer (20 mM Tris, 5 mM EDTA, pH 7.5), and the plate densities were read on a Bio Tek Microplate Reader at 405 nm. The well coated with BSA served as the reagent

blank, and its absorbance was subtracted from that of the other wells. Statistical comparisons among treatment groups were performed using Kruskal-Wallis one-way analysis of variance on ranks (ANOVA: $p < 0.05$). Multiple means comparisons were performed using the Student-Newman-Keuls method (37).

Results

Induction of Vitellogenin in Turtles

A protein of 214 kDa was extractable from E_2 , DES, and both o,p' -DDT treatment groups. This protein exhibited cross-reactivity with antivitellogenin antibodies in Western blots (Figure 1) and was identified as vitellogenin. ELISA analyses of day 14 turtle plasma indicated significant ($p < 0.001$) induction of vitellogenin production from E_2 , DES, and both DDT treatments (Figure 2). E_2 and DES, a well known artificial estrogen, induced the highest concentrations of vitellogenin. The persistent pesticide o,p' -DDT induced smaller amounts of vitellogenin in a dose-dependent manner. No vitellogenin was extractable from the control plasma or detectable using 1D-SDS-PAGE and Western blot analyses, indicating that the slight absorbance of control plasma in ELISA analyses is attributed to nonspecific binding of the polyclonal antibody (Figure 2).

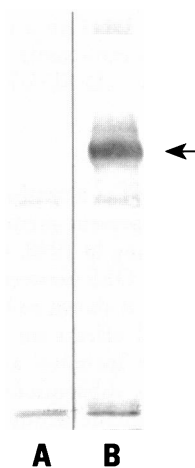


Figure 1. Western blot of plasma from a 1D-SDS-PAGE of control (lane A) and estrogen-treated (lane B) male turtles (*Trachemys scripta*) using antivitellogenin antibody. A protein of 214 kDa (arrow), vitellogenin, is present in plasma of estrogen-treated specimens, but not in controls.

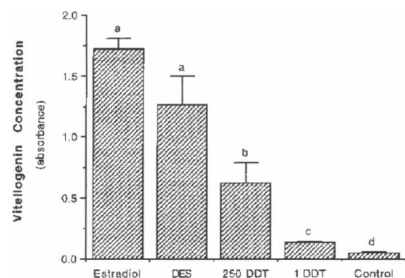


Figure 2. Vitellogenin induction in male turtles treated for 7 days with 1 µg/g estradiol-17β, 1 µg/g DES, 250 µg/g *o,p'*-DDT, or 1 µg/g *o,p'*-DDT. Plasma was collected on day 14 of treatment. Vitellogenin was measured using ELISA with purified polyclonal antibody to turtle (*T. scripta*) vitellogenin. Treatments are significantly different at $p < 0.001$. Treatment groups with different letter designations are statistically different by the Student-Newman-Keuls method.

Induction of Vitellogenin in Frogs

A protein of approximately 200 kDa was extractable from the plasma of frogs treated with E_2 , DES, and both DDT regimes (Figure 3), but was not extractable from the plasma of control specimens. This protein was purified as described above and identified as vitellogenin. However, this protein demonstrated no cross-reactivity with the antiturtle vitellogenin antibodies. Computerized image analysis of 1D-SDS-PAGE was used to quantify relative concentrations of vitellogenin induced by the treatment regimes. The pattern of vitellogenin production in frogs mirrored that seen in turtles, with significant variation ($p < 0.001$) in relative quantities of vitellogenin produced by treatments (Figure 4). E_2 induced the most vitellogenin, followed by DES and *o,p'*-DDT in a dose-dependent fashion. No extractable vitellogenin was detectable by 1D-SDS-PAGE from control samples.

Discussion

DES was developed as a synthetic estrogen that was used to prevent premature births in women beginning in 1948. Over 1 million women took DES between 1960 and 1970 (1). DES was shown to have significant detrimental effects on embryonic development in humans and animal models, and was subsequently banned from use by pregnant women in 1971. Daughters of women who took DES suffer reproductive organ dysfunction, reduced fertility, abnormal pregnancies (39), and increased incidence of vaginal clear-cell adenocarcinomas (40). Similar reproductive and developmental problems have been seen in female laboratory animals (39,41–43). Exposure of male humans and

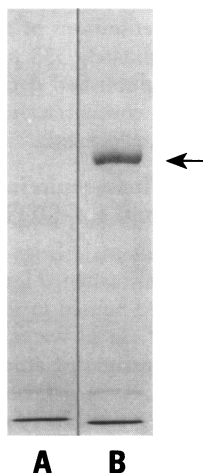


Figure 3. Coomassie blue-stained 5% 1D-SDS-polyacrylamide gel of vitellogenin extracted from plasma from control (lane A) and DDT-treated (lane B) male frogs (*Xenopus laevis*). Induction of 200 kDa protein, vitellogenin (arrow), in males is present in plasma of estrogen-treated specimens, but not in controls.

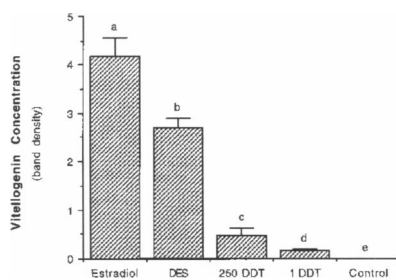


Figure 4. Vitellogenin induction in male frogs (*Xenopus laevis*) treated for 7 days with 1 µg/g estradiol-17β, 1 µg/g DES, 250 µg/g *o,p'*-DDT, or 1 µg/g *o,p'*-DDT. Plasma was collected on day 14 of treatment. Vitellogenin concentration was quantified from 1D-SDS-PAGE by computerized image analysis. Treatments are significantly different at $p < 0.001$. Treatment groups with different letter designations are statistically different by Student-Newman-Keuls method.

male laboratory animals to DES during development leads to lesions of the reproductive system (44,45) and an increased incidence of reproductive tract cancers in laboratory animals (46). The mechanism of DES action has been well studied. It is an agonist of estrogen receptors, binding to them and eliciting transcription of estrogen-induced proteins (39,47).

The use of the organochlorine pesticide DDT was restricted in the United States in 1972; however, elevated levels of DDT and its metabolites are still encountered in the tissues of wildlife in this country (48). Although some of this can be due to

DDT's long half-life (57.7 years) in temperate soils (49), this does not entirely account for the continuing high levels. A likely source is the continued manufacture and use of DDT in developing countries (1). DDT used in developing countries is readily vaporized and transported globally via the atmosphere (50,51), providing a continuing rain of DDT throughout the world. This indicates that DDT is still an important environmental hazard and serves as a good model for physiological studies due to the extensive studies on its mechanism of action.

DDT is well known for its effects on reproduction in avian species, most notably causing eggshell thinning (52). However, the role of DDT as an estrogen has been more insidious (53). DDT has two isomers, *p,p'*-DDT and *o,p'*-DDT, which break down into their respective metabolites, *p,p'*-DDE and *o,p'*-DDE (53). The *o,p'* isomers of DDT and DDE bind to estrogen receptors agonistically, mimicking endogenous estrogen (54,55).

The induction of vitellogenin by a compound indicates that it is an agonist of hepatic estrogen receptors, stimulating them to induce transcription of the vitellogenin genes. Both the artificial estrogen, DES and the pesticide DDT were able to stimulate male turtles and frogs to produce significant levels of vitellogenin. DES has been shown to bind to nuclear estrogen receptors in turtles, inhibiting 96.7% of specifically bound E_2 (56). In the environment, DDT is known to accumulate in large concentrations in turtles and frogs. The softshell turtle has been recorded with 732 ppm DDT in adults (57). Adult toads have exhibited 0.13 ppm DDT (57).

An insidious aspect of lipophilic compounds is that they are also deposited in eggs during vitellogenesis. It is likely that as lipids are mobilized from body stores, lipophilic contaminants also are mobilized and become incorporated into the growing ovarian follicles. Thus the adult's burden of lipophilic contaminants may be passed on to their offspring, where they can influence the fertilizability of the egg and development of the embryo, the most sensitive period of the life cycle. Eggs of loggerhead sea turtles exhibited approximately 0.1 ppm DDE (58), equal to the highest levels detected in the adult turtles (59). In the snapping turtle, up to 864 ppb DDT has been reported in their eggs (60). The levels of organochlorine contaminants in reptilian eggs correlates with increases in deformities and lowered rates of hatching success (61). In species that

exhibit temperature-dependent sex determination (TSD), such as many turtles, the implications for effects by environmental estrogens are enormous. The sex of the offspring in species that exhibit TSD is regulated by levels of sex steroids (62,63). By increasing levels of perceived estrogen, xenobiotic estrogens may adversely affect sexual development in these species. It has been shown that exposure of turtle eggs to xenobiotic estrogens induces ovary-like characteristics in hatchlings incubated at male-producing temperatures (64).

Vitellogenin may prove useful as a biomarker for exposure to xenobiotic estrogens due to its numerous advantages. First, it is applicable to a wide range of species, potentially any oviparous or ovoviparous vertebrate. The criterion for using vitellogenin as a biomarker in a particular species is that males do not normally express vitellogenin, but are capable of doing so in response to estrogenic compounds. Second, the test is relatively non-invasive, requiring only small (microliter) quantities of plasma or serum. This eliminates the necessity of acquiring tissue biopsies of organs such as liver, muscle, and fat. Third, it indicates a physiological response to an environmental challenge, indicating that the animal is being influenced by environmental estrogens. This eliminates the confusion often encountered when contaminants are detected in wildlife, but without knowing if the contaminant is causing any physiological effects. Fourth, the expression of vitellogenin is through known physiological and biochemical pathways. Indeed, the induction of vitellogenin has been studied intensively as a model for steroid regulation of gene expression and protein synthesis (4–13). Fifth, the induction of vitellogenin is sensitive to any estrogenic contaminant. In fact, the vitellogenic response has been used to screen unknown compounds for

estrogenic properties (29,30). Sixth, the response is quantifiable. Finally, the assay for vitellogenin can be performed relatively easily and inexpensively, especially compared to the exorbitant cost of screening for a wide range of specific contaminants. The vitellogenic response has been used to test for exposure to estrogenic xenobiotics in fishes (26–30).

There are, however, several drawbacks regarding vitellogenin analysis that must be considered. It is unlikely that the production of vitellogenin in males represents a seriously deleterious physiological response. This assay also provides no direct information regarding the female or developing embryo. However, if estrogen receptors are being stimulated in the liver of males, receptors in other organs such as the testes and prostate gland of males and reproductive tissues of females and embryos may likewise be affected. In fact, since females and embryos often show greater responses to estrogen, they may be affected equally or even more so. Further, vitellogenin production does not indicate what compound may be causing the effect. However, it may be used as a rapid, sensitive, and economical initial screen, followed (as indicated by positive vitellogenic responses) by more costly screens to identify the specific contaminating compounds. This assay assumes that males do not produce vitellogenin but are capable of doing so. This requires that some physiology of the species be known, such as comparing contaminated populations to clean control populations. These limitations considered, vitellogenin may serve as an excellent biomarker for xenobiotic estrogen exposure in reptiles and amphibians or other oviparous and ovoviparous vertebrates.

Increasingly more evidence of the estrogenic effects of contaminants on wildlife is appearing. In the late 1970s, male herring

gull embryos and chicks from Lake Ontario showed the development of oviducts and ovarylike gonads. Laboratory studies have since shown that estrogenic pesticides such as dicofol, kelthane, and methoxychlor produce these effects in California gulls, western gulls, and kestrels (65). Herring gull populations contaminated with estrogenic compounds such as DDT, DDE, and PCBs have been shown to form female-female pairs (66). In Florida, the occurrence of demasculinized and feminized male alligators is associated with the presence of the estrogenic pesticides (67). Hermaphroditic fish populations have been reported in the lagoons of sewage treatment works (27).

Are these examples of wildlife effects isolated incidents or just the tip of the iceberg? It is unclear at this point. Some populations, such as bald eagles surrounding the Great Lakes, are still in trouble. Adult bald eagles that migrate to the lake shore develop reproductive difficulties associated with contaminated food (48). The Great Lakes is acting as a sink for bald eagles migrating from reproductively fit inland populations. Alligators in Lake Apopka, Florida, have drastically reduced reproductive success (67,68). These local populations of long-lived species may be heading for extinction, because reproduction on the site is insufficient to maintain the population size, requiring migration from surrounding populations to sustain them. It is possible that the decline of other species around the globe, such as amphibians (69–74), is partially linked to reproductive impairment by endocrine-disrupting compounds (BD Palmer, unpublished data). The assay of vitellogenin in males of these and other species in the wild would indicate whether they are being physiologically influenced by the estrogenic properties of environmental contaminants.

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